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Efficacy of oxytetracycline and potentiated sulphonamide oral therapies against *Aeromonas hydrophila* infection in Nile tilapia *Oreochromis niloticus*

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ABSTRACT

Objective: To assess the efficacy of feeds containing approved antibiotics, *viz.*, oxytetracycline (OTC) or potentiated sulphonamide [(sulphamethoxazole-trimethoprim) SMZ-TMP] at 1 g, 2 g, 3 g and 4 g of respective antibiotics/kg feed at 2% body weight ration in preventing the *Aeromonas hydrophila* (*A. hydrophila*) infection in *Oreochromis niloticus*.

Methods: Commercial pellet feed was top dressed with respective antibiotics using 5 mL vegetable oil as a binder. Fish were injected intramuscularly with *A. hydrophila* at ≈ 6.0×10^7–8.6×10^7 CFU/fish, and fed subsequently with OTC and SMZ-TMP feeds for 10 and 5 days, respectively. Fish mortalities were recorded during the pre-treatment, treatment and post-treatment periods.

Results: Highest mortalities (7.42%–8.33%) were observed in challenged and untreated fish. The mortalities observed in fish fed with OTC or SMZ-TMP were 0%–6.66% with decreasing concentrations of antibiotics from 4 to 1 g/kg feed. Significant differences existed in the mortalities among fish fed with different doses of antibiotics ($P < 0.05$). The relative percent survival values were 20, 40, 40 and 60 for 1 g, 2 g, 3 g and 4 g OTC/kg feed groups, respectively; while in SMZ-TMP fed fish, the respective relative percent survival values were 10, 100, 100, and 100.

Conclusions: The fish fed with feed containing 2 g antibiotic/kg at 2% body weight was the lowest concentration that recorded significantly lower mortality ($P < 0.05$), which could be the treatment of choice for the control of *A. hydrophila* in *Oreochromis niloticus* in tropical condition.

1. Introduction

Tilapias are the world's second most important fish species for aquaculture after the carp. Tilapias have long been considered as an ideal species for use in aquaculture due to their market demand, high growth rate, hardy nature, tolerance of suboptimal water quality, and disease resistance[1]. Nile Tilapias are the predominant cultured tropical species that prefer to live in shallow water. The preferred temperature for Nile tilapia growth ranges from 31 to 36 ˚C[2]. Disease outbreaks were recently identified as a major constraint to tilapia production and trade, with consequent effect on the industry's economic development[3,4]. Bacterial diseases have become major barriers to aquaculture, especially when the water temperature is warm. *Aeromonas hydrophila* (*A. hydrophila*) is the most prevalent during the new season culture where it infects not only fish, but human are also susceptible to infection[5]. It is the causative agent of motile aeromonad septicemia (MAS). The symptoms of MAS include swelling of tissues, dropsy, red sores, necrosis, ulceration and haemorrhagic septicemia[6-8]. The presence of *A. hydrophila*, by itself, is not indicative of disease and, consequently, stress is often considered to be a contributing factor in the outbreak of disease caused by this bacterium[5]. Elevated water temperature, decreased dissolved oxygen concentration, or increased ammonia and carbon-dioxide concentrations have been shown to promote stress in fish and trigger MAS[6,7].

Antimicrobial agents have been widely used in aquaculture

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worldwide to treat infections caused by a variety of bacterial pathogens of fish. Oxytetracycline (OTC) and potentiated sulphonamide [(sulphamethoxazole-trimethoprim) SMZ-TMP] are the two approved antibiotics, which are widely used in aquaculture industry[7,9,10]. These antibiotics are used to treat diseases, but only in certain types of aquatic animals (channel catfish, salmonids and lobster with OTC) and only to treat certain diseases[7,9,10]. The use and abuse of antibiotics in aquaculture has increased the selective pressure exerted on the microbes and encouraged the emergence of resistant bacteria by transferring resistant genes to bacteria not exposed to antibiotics. Moreover, the antimicrobials lead to drug residues in the treated fish, besides having a negative impact on the environment[9,11]. The effectiveness and safety levels of the Food and Drug Administration approved antibiotics with temperate fish have been established[10]. However, such studies on fish species cultured in tropical conditions are not available. This study was, therefore, aimed at to evaluate the effectiveness of medicated feeds containing varied concentration of OTC and SMZ-TMP on Nile tilapia *Oreochromis niloticus* (*O. niloticus*) against *A. hydrophila* infection in tropical condition.

2. Materials and methods

2.1. Bacterial strain and experimental fish

The β-haemolytic bacterial strain *A. hydrophila* BBT4K3, (NCBI accession number KY484791) isolated from the kidney of haemorrhagic septicemic Nile tilapia *O. niloticus*, was from the collections of the Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, India. The identity of this strain was reconfirmed phenotypically by VITEK 2 compact system (bioMérieux, France). The strain was sensitive to OTC and ormetoprim.

Healthy *O. niloticus* juveniles [(8.9 ± 0.7) cm; (8.2 ± 0.5) g] were brought from Naihati, North 24 Parganas district, West Bengal, India in oxygen filled polyethylene bags to Faculty of Fishery Sciences, Kolkata as requirement. The fish were acclimatized for 3 h followed by disinfection with 5 mg/mL potassium permanganate for 15 min. The juveniles were stocked in 500 L capacity fiberglass reinforced plastic tanks at 75 numbers/tank containing 400 L clean bore well water. The fish were acclimatized for about two weeks and fed *ad libitum* with commercial pellet basal feed containing 30% crude protein (CP Pvt. Ltd., India) at 2% body weight.

2.2. Preparation of medicated feeds

As per the Food and Drug Administration, the approved dose of OTC is 2.50–3.75 g/100 pounds body weight/day (or 55–83 mg/kg biomass/day) for 10 consecutive days and the dose of SMZ-TMP is 50 mg/kg body weight/day for 5 consecutive days[10]. The OTC and SMZ-TMP medicated feeds were prepared for feeding the fish at 2% body weight. Briefly, the feeds were prepared by first mixing 1 g, 2 g, 3 g and 4 g of OTC dihydrate (HiMedia, India) and SMZ-TMP (Bactrim D.S., Abbott Healthcare Pvt Ltd., Mumbai, India) separately in 5 mL vegetable oil, and then admixed with 1 kg basal feed in airtight plastic containers. After proper mixing, the medicated feeds containing varied concentration of OTC and SMZ-TMP were uniformly spread, dried under the fan for 24 h, and stored in airtight plastic containers at room temperature (24–28 °C).

2.3. Bacterial challenge and evaluation of efficacy of oral antibiotic therapies

The experiments were carried out in plastic tanks of size L58 cm × H45 cm × B45 cm with *O. niloticus* juveniles in triplicate. For experimental trials with OTC, the fish were divided into 7 groups, namely, Group 1: negative control (unchallenged and fed with basal feed), Group 2: positive control (saline injected, unchallenged and fed with basal feed), Group 3: 0 g OTC/kg feed, Group 4: 1 g OTC/kg feed, Group 5: 2 g OTC/kg feed, Group 6: 3 g OTC/kg feed, and Group 7: 4 g OTC/kg fish. The healthy juveniles were stocked in tanks containing 80 L water (20 fish/tank). A similar experimental design was followed for the trials with SMZ-TMP. After 5 days of acclimatization in tanks, the fish were injected intramuscularly at the base of the dorsal fin. Prior to challenge, the fish were starved for a day and anesthetized using clove oil at 50 μL/L water. The concentrations of *A. hydrophila* used for the challenge were 1×10^8 cells/mL for OTC feed trials and 8.6×10^7 cells/mL for SMZ-TMP feed trials. The fish of OTC feed groups were fed with respective OTC feeds twice daily at 2% body weight for 10 days; while those of SMZ-TMP feed groups were fed with respective SMZ-TMP feeds twice daily at 2% body weight for 5 days[10]. The unconsumed feed, if any, in each tank was removed daily, air dried and weighed carefully. Observations on the feeding behaviour, behavioural changes, external signs of infections and mortalities were recorded during the pre-treatment period (Days 1–5), disease progression period (Days 6–8), treatment period (Days 9–18 for OTC group and Days 9–13 for SMZ-TMP group) and post-treatment period (21 days: Days 19–39 for OTC group and Days 14–34 for SMZ-TMP group). Depending on the feed consumption, the feeding behaviour of tilapia was rated using a scale ranging from 0 to 4, *i.e.*, 4: 100% feed consumption, 3: 75% feed consumption, 2: 50% feed consumption, 1: 25% feed consumption and 0: no feed consumption. The freshly dead tilapia juveniles of OTC or SMZ-TMP oral therapy trials were subjected to necropsy and bacteriology. Inocula from the haemorrhagic area and kidney of fish were streaked onto Rimler-Shotts agar plates and incubated at 35 °C for 24–48 h[6,7]. The efficacy of OTC and SMZ-TMP oral therapy was evaluated by calculating the relative percent survival (RPS)[7] for both OTC and SMZ-TMP feed treatments as given below.

$$\text{RPS} = \left(1 - \frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}}\right) \times 100$$

2.4. Statistical analysis

The results of each experiment are expressed as mean ± SD and analyzed by One-way ANOVA to test the significance of difference between the control and experimental groups. Comparison of mean

values was done by Duncan's multiple range test[12]. A probability level of 0.05 was used to find out the significance in all cases.

3. Results

The cumulative mortalities in *O. niloticus* juveniles challenged with *A. hydrophila* and fed OTC feed for 10 days are presented in Figure 1. On Day 1 post-injection, 3.33% mortality was observed in 1 g OTC/kg feed group and no mortalities were noticed in all other tanks. During the disease progression period, *i.e.*, on Day 8 post-challenge, 3.33%–8.33% mortalities were noticed in treatment groups 3–7. At the end of 10 days OTC treatment regime, *i.e.*, on Day 19 post-challenge, the mortalities observed in *A. hydrophila* challenged and OTC fed fish were 3.33%–6.66%. The positive and negative controls recorded 3.33% and no mortalities, respectively. The highest mortality (8.33%) was observed in the challenged and control feed fed fish. The RPS values were 20.05, 39.98, 39.98 and 60.02 for 1 g OTC/kg feed, 2 g OTC/kg feed, 3 g OTC/kg feed, 4 g OTC/kg feed groups, respectively.

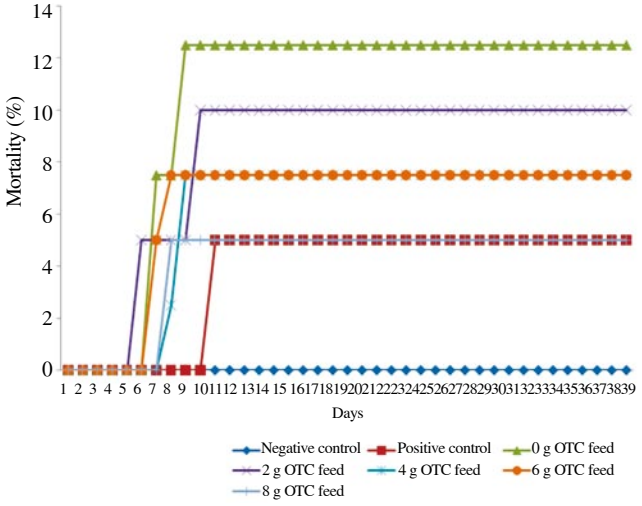

Figure 1. Cumulative mortalities in *O. niloticus* juveniles challenged with *A. hydrophila* and fed OTC feed for 10 days.

The cumulative mortalities in *O. niloticus* juveniles challenged with *A. hydrophila* and fed SMZ-TMP feed for 5 days are presented in Figure 2. During the disease progression period, 0%–3.94% mortalities were noticed. At the end of 5 days SMZ-TMP treatment regime, *i.e.*, on Day 13 post-challenge, the mortalities observed in *A. hydrophila* challenged and SMZ-TMP fed fish were 0%–5.88%. The highest mortality (7.82%) was observed in challenged control fish. The RPS values were 9.95 for 1 g SMZ-TMP/kg feed and 100 for 2–4 g SMZ-TMP/kg feed groups. There existed significant differences ($P < 0.05$) in the mortalities among fish fed with different doses of two antibiotics (Figure 3). The differences in mortalities of fish fed with OTC and SMZ-TMP feeds at lower concentrations were insignificant ($P > 0.05$). Internally, discoloration and liquefaction of the internal organs such as kidney and liver were observed in challenged fish. Bacteriological samples taken from the fish kidney confirmed *Aeromonas* infection as revealed by the exclusive growth of yellow colour bacterial colonies on Rimler-Shotts agar plates at 35 ˚C and standard biochemical test results.

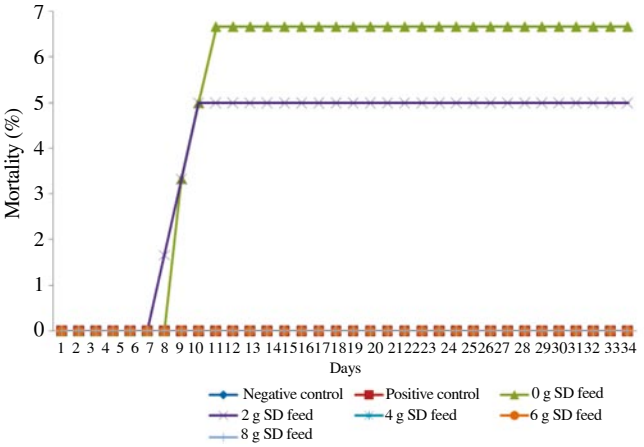

Figure 2. Cumulative mortalities in *O. niloticus* juveniles challenged with *A. hydrophila* and fed sulpha drug (SD) feed for 5 days.

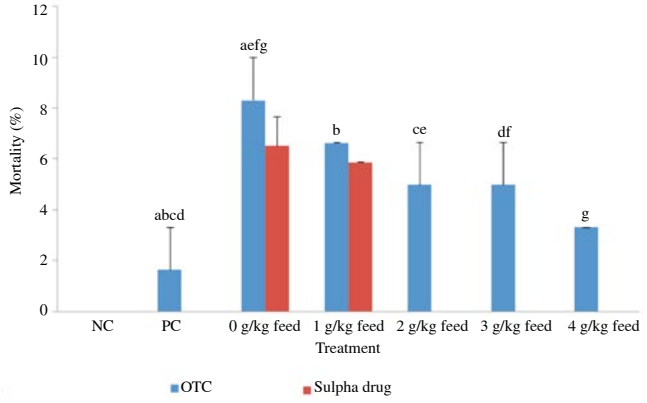

Figure 3. Mortalities in *A. hydrophila* challenged *O. niloticus* juveniles when fed with OTC or sulpha drug supplemented feeds.
[a-g]: Bars sharing common superscripts differ significantly ($P < 0.05$); PC: Positive control (saline injected); NC: Negative control.

4. Discussion

The results of the present study demonstrated the efficacy of oral OTC and SMZ-TMP therapies (1–4 g OTC or SMZ-TMP/kg feed) following *A. hydrophila* challenge in *O. niloticus*. Antimicrobial drugs are powerful tools for the management of infectious diseases in aquatic animals. Most of the drugs legally used in aquaculture were approved by the government agency responsible for veterinary medicine, for example, the United States Food and Drug Administration in the USA. For instance, in the USA, OTC, florfenicol, and sulfadimethoxine/ormetoprim are authorized for use in aquaculture, only in certain types of aquatic animals and only to treat certain diseases. The regulatory agencies may set rules for antibiotic use, including permissible routes of delivery, dosage forms, withdrawal times, tolerances, and use of species, including dosage rates and limitations[11]. The most common route for the delivery of antibiotics for fish occurs through mixing the antibiotic with specially formulated feed. Following *A. hydrophila* challenge and oral therapy, a reduction in tilapia mortality with increasing concentrations of antibiotic was observed. Salte and Liestol[13] also

demonstrated that the fish treated with OTC at a dose of up to 75 mg/kg of fish body weight for 10 consecutive days have a withholding period of 60 days at water temperatures above 10 ℃ and of 100 days at water temperatures between 7 and 10 ℃. They further noted that fish treated with potentiated sulfonamides (sulfadiazine + trimethoprim) at a dose of up to 30 mg/kg of fish body weight for 10 consecutive days, have a withholding period of 60 days at water temperatures above 10 ℃.

A. hydrophila challenge caused fish mortalities only during the disease progression and early OTC treatment periods. The lowest mortality was noted as 3.33% in 4 g OTC/kg feed group, followed by 5% each in 2 g and 3 g OTC/kg feed groups and no mortality in 2 g, 3 g and 4 g SMZ-TMP/kg feed groups. In both the cases, the difference in mortalities between 0 g and 1 g antibiotic/kg feed groups was insignificant ($P > 0.05$). Significant difference in mortalities existed between 1 g and 4 g antibiotic/kg feed groups. There existed significant differences among 0 g antibiotic/kg feed group and the other antibiotic feed fed groups such as 2 g, 3 g and 4 g antibiotic/kg feed. These results further suggested that the feeds with OTC or SMZ-TMP at 2–4 g antibiotic/kg feed at 2% body weight ration is ideally used to control *A. hydrophila* infection in Nile tilapia. Likewise, Plumb[3] recommended a medicated feed with 2–4 g OTC/kg feed (50–100 mg/kg fish) for 14 days for bacterial disease treatment. The highest RPS (60.02) was recorded in 4 g OTC/kg feed group. The RPS recorded in 2 g, 3 g and 4 g SMZ-TMP/kg feed groups were 100. It was opined that high levels of antibiotic (OTC) may even fail sometimes to eradicate the bacteria, leading to the conclusion that the bacterial strain in the host is resistant to the antibiotic[14]. Therefore, antibiotic therapy must be exercised with caution.

Although using medicated feeds is one of the easiest ways to treat fish, they must be used early and quickly because sick fish frequently will stop feeding[15]. In the present study also there was a reduction in medicated feed consumption in all the experimental groups when challenged with *A. hydrophila*. The feeding rates and the feeding behaviour scores (2.6–3.0) were the minimum during the disease progression period, which subsequently increased to almost normal (3.9–4.0) at the end. The wounds of OTC or SMZ-TMP fed groups at the site of injection healed faster than the control groups. The present study, thus, demonstrated the effectiveness of oral antibiotic therapy in reducing the *A. hydrophila* induced mortalities in Nile tilapia, which was found to be dose-dependent. The results further suggested the prudent use of 2–4 g antibiotic/kg feed to control *A. hydrophila* infection in Nile tilapia as outlined by Food and Agriculture Organization[9,11]. The information generated in this study would help to ensure that responsible antibiotic therapy retains its place as a rapid and decisive aid, which has made it efficacious up to now.

Conflict of interest statement

We declare that we have no conflict of interest.

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